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The sensitivity of AMPA-selective glutamate receptor channels to pentobarbital is determined by a single amino acid residue of the α2 subunit

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Abstract Clinical concentrations of pentobarbital inhibit the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective glutamate receptor (GluR) channels. Recently, the AMPA-selective GluR channels that contained the α2 subunit were shown to be more sensitive to pentobarbital block than those without the α2 subunit. Here we demonstrated that replacement by glutamine of the arginine residue in putative transmembrane segment M2 of the α2 subunit (mutation α2-R586Q) drastically reduced the pentobarbital sensitivity of the α2 heteromeric channel to the level comparable to those of the α1 and α2-R586Q homomeric channels. These results suggest that the arginine residue in segment M2 of the α2 subunit is the critical determinant of the sensitivities of the AMPA-selective GluR channels to pentobarbital.

Key words: Pentobarbital; AMPA-selective glutamate receptor channel; α2 Subunit; Site-directed mutagenesis; Anesthetic

1. Introduction

Barbiturates are clinically used for their potent anesthetic, hypnotic, sedative and anticonvulsant actions. In various neurophysiologic systems, barbiturates were shown to enhance the synaptic actions of inhibitory neurotransmitters, such as γ-aminobutyric acid (GABA) [1]. Other studies, however, reported that barbiturates inhibited the neuronal responses to excitatory neurotransmitters. For example, the sensitivity of cortical neurons to L-glutamate was depressed by barbiturates [2]. Furthermore, pentobarbital block of kainate currents in cultured mouse hippocampal neurons was shown to be voltage-dependent [3] and that of kainate and quisqualate currents in cultured rat cortical neurons was use-dependent [4]. These observations suggest the channel block mechanisms of barbiturates action to inhibit the glutamate receptor (GluR) channels.

Cloning and expression studies have demonstrated that the members of the α (GluR1–4 or GluRA-D) subfamily of the GluR channel subunits represent the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective GluR channel [5–7], the members of the β (GluR5–7) and γ (KA) subfamilies constitute the kainate-selective GluR channel [6,7] and the ε (NR2) and ζ (NR1) subfamilies represent the *N*-methyl-D-aspartate (NMDA) receptor channel [6,7]. Recent studies have found that the sensitivities of the AMPA-selective GluR channels to pentobarbital vary with subunit composition and that the α2 subunit makes the GluR channels more sensitive to pentobarbital [8]. The α2 subunit possesses a unique positively charged amino acid residue (arginine-586) in putative transmembrane segment M2 which determines the Ca^{2+} permeability of the AMPA-selective GluR channels [9–11]. To reveal the mechanisms of action of barbiturates on the AMPA-selective GluR channels, we examined the effects of replacement by glutamine of the arginine residue in segment M2 of the α2 subunit on the sensitivities of the AMPA-selective GluR channels to pentobarbital.

2. Materials and methods

Subunit-specific mRNAs were synthesized in vitro with SP6 RNA polymerase (Ambion MEGAscript) in the presence of cap dinucleotides ^{7}mGpppG. The α1, α2 and α2-R586Q subunit-specific mRNAs were synthesized as described [5,10], except that the α1 subunit mRNA used for the expression of the α1 homomeric channel was synthesized from the plasmid pSPTGRα1 [12]. *Xenopus laevis* oocytes were injected with the α1 or α2-R586Q subunit-specific mRNA or with the α1 subunit-specific mRNA and the α2 or α2-R586Q subunit-specific mRNA in a molar ratio of 10:1; the concentrations of respective mRNAs were 200 ng/ml when expressed alone and 200 ng/μl and 20 ng/μl for 10:1 combinations and the average volume injected was ~50 nl per oocyte. After incubation at 20°C for 2–3 days, whole-cell currents evoked by bath-application of 100 μM kainate for ~15 s were recorded at −70 mV membrane potential and ~19°C in normal frog Ringer's solution with a conventional two-micropipette voltage clamp [5]. The effects of pentobarbital on the GluR channels were examined by measuring current responses evoked by the second application of agonist during pentobarbital perfusion. The effect on the α2 homomeric channel was not investigated because of its low activity [5]. Normal frog Ringer's solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES–NaOH (pH 7.2).

3. Results and discussion

Recently, the AMPA-selective GluR channels that contained the α2 subunit (the α1/α2 and α2/α3 channels) were shown to be more sensitive to pentobarbital block than those without the α2 subunit (the α1, α3 and α1/α3 channels) [8]. Although the amino acid sequences in the putative transmembrane segment M2 are highly conserved among the a subunit species (α1–α4), only the α2 subunit contains the unique positively charged amino acid residue (arginine-586) in segment M2 (Fig. 1) [5]. To examine whether this arginine residue of the α2 subunit was

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; GABA, γ-aminobutyric acid; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate.

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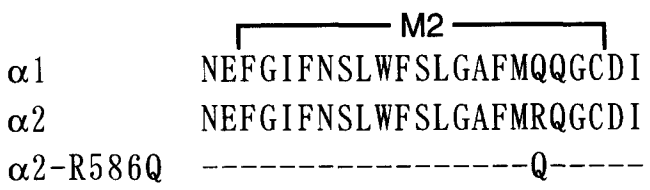

Fig. 1. The substitution mutation introduced into the α2 subunit of the mouse AMPA-selective GluR channels. Amino acid sequences of the putative transmembrane segment M2 are shown for the α1, α2 and α2-R586Q subunits. Bars represent residues identical to those above in the aligned position.

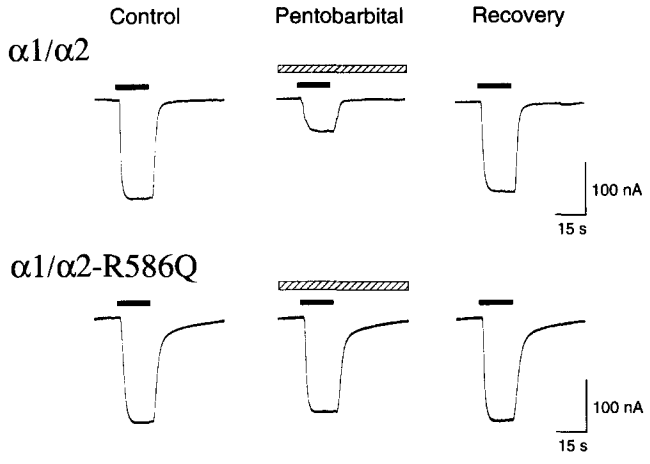

Fig. 2. Current responses of the α1/α2 and α1/α2-R586Q channels before (left), during (middle) and after (right) perfusion of 100 μM pentobarbital. Inward current is downward. The duration of 100 μM kainate application is indicated by bars without taking into account the dead-space time in the perfusion system (~2 s). The duration of pentobarbital perfusion is indicated by hatched column.

involved in the higher pentobarbital sensitivities of the AMPA-selective GluR channels with the α2 subunit, effects of replacement by glutamine of the arginine residue of the α2 subunit (mutation α2-R586Q; Fig. 1) on the sensitivities of the α1/α2 channel to pentobarbital were examined.

The α1/α2 and α1/α2-R586Q heteromeric channels and the α1 and α2-R586Q homomeric channels were expressed in *Xenopus* oocytes by the injection of respective subunit-specific mRNAs synthesized in vitro from cloned cDNAs. Fig. 2 shows current responses of the α1/α2 and α1/α2-R586Q channels to kainate before, during and after perfusion of 100 μM pentobarbital. Pentobarbital effectively suppressed the current response of the α1/α2 channel. After washout of pentobarbital, the current response of the α1/α2 channel was almost fully recovered. On the other hand, the current response of the α1/α2-R586Q channel was only slightly suppressed by pentobarbital in a reversible manner. The dose–inhibition relationships for pentobarbital of the α1, α1/α2, α1/α2-R586Q and α2-R586Q channels were examined (Fig. 3). The current responses of the α1/α2 channel were reduced to 50% by ~45 μM pentobarbital and were almost completely suppressed by 1000 μM pentobarbital. On the other hand, the replacement by glutamine of the arginine residue in segment M2 of the α2 subunit drastically reduced the sensitivity of the α1/α2 heteromeric channel to the level comparable to those of the α1 and α2-R586Q homomeric channels. The IC_{50} (inhibitor concentration for half-control response) value of the α1/α2-R586Q channel was ~880 μM which was close to those of the α1 and α2-R586Q channels (~1070 and ~1080 μM, respectively).

The α2 subunit is a key molecule that endows the heteromeric AMPA-selective GluR channels with functional properties of native AMPA receptor channels [13]. The α2 subunit also appears to be important with respect to its contribution to the pentobarbital sensitivity of native AMPA receptor channels because the IC_{50} value for the pentobarbital inhibition of kainate currents in cultured cortical neurons (50 μM) [4] is similar to that of the α1/α2 heteromeric channel (~45 μM). The anesthetic EC_{50} (plasma concentration to prevent movement of 50% of patients in response to a painful stimulus) value for pentobarbital is ~50 μM [1]. Thus, the AMPA-selective GluR channels may be considered to be a potential anesthetic target for pentobarbital. Drastic reduction in the pentobarbital sensitivity of the α1/α2 channel caused by the mutation α2-R586Q suggests that the arginine residue in segment M2 of the α2 subunit is the critical determinant of pentobarbital sensitivities of the AMPA-selective GluR channels. Since pentobarbital is neutral or perhaps negatively charged [3] and the arginine residue is positively charged, it may be reasonable to assume some interaction between these molecules.

Site-directed mutagenesis showed that the arginine-586 in segment M2 of the α2 subunit determines the Ca^{2+} permeability of the AMPA-selective GluR channels [9–11]. In the present investigation, we have shown that the same arginine residue is also a critical determinant of the sensitivities of the AMPA-selective GluR channels to pentobarbital. Similarly, we have previously shown that the conserved asparagine residue in segment M2 of the ε2 and ζ1 subunits (at the position corresponding to the arginine-586 in segment M2 of the α2 subunit),

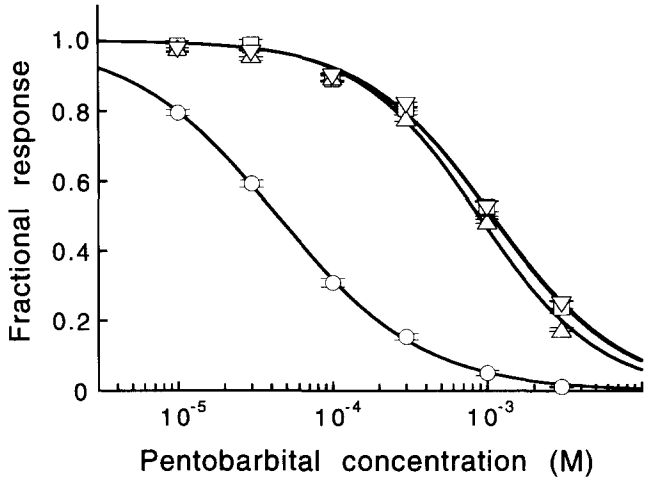

Fig. 3. Effects of the substitution mutation on the sensitivities to pentobarbital. The dose–inhibition relationships for pentobarbital of the α1 (□), α1/α2 (○), α1/α2-R586Q (△) and α2-R586Q (▽) channels were examined. Each point represents the mean ± S.E.M. of measurements on 6–11 oocytes; S.E.M. are indicated by bars when larger than the symbols. The theoretical curves have been drawn according to the equation: $I_{pen} = I_{con}/[1 + (P/IC_{50})^n]$, where I_{pen} represents the current response during pentobarbital perfusion, I_{con} the control current response, P the concentration of pentobarbital, and n the Hill coefficient. The control current responses obtained before pentobarbital perfusion were 75–350 nA (□), 89–431 nA (○), 120–725 nA (△) and 100–600 nA (▽).

constitutes a Mg^{2+} block site of the NMDA receptor channels [14], and that non-competitive antagonists, for example dissociative anesthetics, also act on the Mg^{2+} block site [15]. Since clinical concentrations of these general intravenous anesthetics (barbiturates and dissociative anesthetics) act on the crucial sites in channel-forming segment which determine the channel properties, the channel block of ligand-gated ion channels mediating excitatory synaptic transmission may be one of important mechanisms of action of general anesthetics.

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